

Functional Maturation during Bovine Granulopoiesis

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ABSTRACT

Granulocytic precursor cells undergo morphologic changes in the nucleus and the cytoplasm during the process of granulopoiesis, which takes place in the bone marrow. These changes are associated with the development of stage-specific proteins necessary for the highly specialized roles of polymorphonuclear leukocytes in phagocytosis, bacterial killing, and in mediating the inflammatory process. The objective of the current study was to sequence the various events that occur upon functional development of granulocytic bone marrow cells in the bovine species.

Cells were obtained from the bone marrow of clinically healthy cows and separated into different stages of maturation using density gradient centrifugation. Three cellular fractions were obtained that were enriched for either early immature, late immature or mature granulocytic cells. Functions and receptor expressions assessed in the three maturation stages were: Fc-IgG₂ receptor and CD11b expression, phagocytosis of *Escherichia coli*, respiratory burst activity, and cellular myeloperoxidase activity. Immature cells expressed already Fc-IgG₂ receptor and CD11b on their cytoplasmic membrane. Phagocytic ability was acquired in the myelocytic stage, but only the more mature forms were readily capable of phagocytosis. Promyelocytes, myelocytes and metamyelocytes showed no respiratory burst activity. Only band and segmented cells produced reactive oxygen species. Myeloperoxidase was present at all stages of maturity. Thus, each of the maturation stages was characterized by a selective expression of one or more functions and receptors. Therefore, sequential biochemical maturation is postulated during bovine granulopoiesis.

(Key words: bovine, bone marrow, functional maturation, granulopoiesis)

Abbreviation key: FBS = fetal bovine serum, FITC = fluorescein isothiocyanate, FS/SS = forward scatter/

side scatter, F = fluorescence, MFI = mean fluorescence intensity, MPO = myeloperoxidase, PI = propidium iodide, PMA = phorbol 12-myristate 13-acetate, PMN = polymorphonuclear leukocytes, ROS = reactive oxygen species.

INTRODUCTION

It is well known that the neutrophils constitute the most important non-specific defense mechanism when pathogens invade the bovine udder (Burvenich et al., 1994). In infected glands, the percentage of neutrophils in milk approaches 100%. The increased susceptibility to mastitis in high-yielding cows during early lactation is associated with decreased neutrophil function. Kehrli et al. (1989) hypothesized that an immunosuppression may occur during the periparturient period and therefore predisposes the dairy cow to new infections. Several of the altered neutrophil functions were found to be related to the severity of experimentally induced *Escherichia coli* mastitis during early lactation (Heyneman et al., 1990; Lohuis et al., 1990; Kremer et al., 1993; Van Werven et al., 1997). The number of leukocytes prior to infection was also decreased.

The number of circulating polymorphonuclear leukocytes (PMN) is the result of influx and efflux of leukocytes. The efflux depends on the migration of the cells towards the marginated pool and towards tissues. Neutrophils do not recirculate. There is a unidirectional movement from the bone marrow into the blood, and from circulation towards the tissues. A well-controlled equilibrium exists between the circulating and the marginated pool in the blood (Figure 1). Upon demand, a physiological mechanism appears for the rapid release of mature granulocytes into the circulation and accelerates the maturation of cells in the bone marrow (Moreira da Silva et al., 1994). When maturation is impaired, e.g. due to the increased proliferation rate, a higher number of immature cells will appear in circulation. This phenomenon occurs in periods of stress (e.g. parturition, mastitis).

All peripheral blood and immune cells originate from the bone marrow by proliferation and differentiation of a pluripotent stem cell, becoming committed to a particular lineage. Polymorphonuclear leukocytes are

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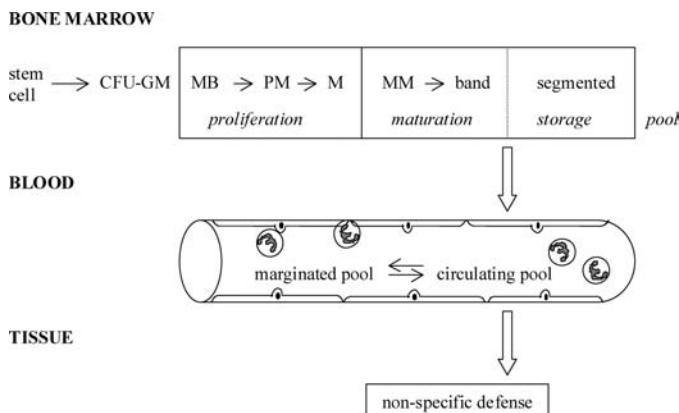


Figure 1. Lifecycle of bovine PMN, according to the data of Jain (1993). A unidirectional progression of proliferation, differentiation and maturation from the stem cell through myeloblast, promyelocyte, myelocyte, band cell and segmented cell results in the formation of mature PMN. Cells are released into circulation and transported to the tissues where they exert their function in the non-specific defense.

(CFU-G = colony-forming unit of granulocytes, MB = myeloblast, PM = promyelocyte, M = myelocyte, band = band cell, segmented = segmented cell).

formed from the colony-forming unit of granulocytes, through the stages of myeloblast, promyelocyte, myelocyte, metamyelocyte, and band cell. Three functional compartments of granulocytes are recognized in the bone marrow covering the granulopoiesis process: (1) the proliferation or mitotic pool, consisting of myeloblasts, promyelocytes, and myelocytes; (2) the maturation or non-mitotic pool with metamyelocytes and band cells; and (3) the storage pool, primarily comprised of mature granulocytes and some band cells (Jain, 1993; Moreira da Silva et al., 1994; Figure 1). Myeloid precursors undergo striking morphologic and functional changes during the process of granulocytic maturation. These changes are associated with significant changes in cell size and nuclear shape, and with the development of stage-specific proteins necessary for phagocytosis, and bacterial killing, and in mediating the inflammatory response (Berliner, 1998). In the human, various biochemical and functional properties of PMN are acquired in a well-controlled stepwise manner during granulopoiesis (Glasser and Fiederlein, 1987). The sequence for the functional differentiation of the human neutrophil has been proposed to be the following: Fc receptors—immune phagocytosis—complement receptors—oxygen-independent microbial killing—oxygen-dependent microbial killing—chemotaxis. If this phenomenon would also occur in the bovine, the appearance of myelocytes, metamyelocytes and band cells in circulation during infection could be involved in the impaired defense during parturition “stress”. Guidry and Paape (1976) also postulated that the left-shift observed dur-

ing mastitis could compromise the cows’ resistance by supplying more cells that are morphologically immature and functionally inadequate.

Until now, bone marrow research in the bovine species was scarce. Recently, Hoeben et al. (1999) provided a first indication of the importance of the granulopoiesis by studying the effect of hormones and metabolites, of which the concentration changes dramatically during the periparturient period, on bovine bone marrow cells in vitro. Acetoacetic acid, β -hydroxybutyric acid, hydrocortisone-21-acetate and bovine pregnancy-associated glycoprotein were found to have inhibitory effects on the proliferation of progenitor cells.

The objective of the present study was to investigate the maturation sequence of the functional properties of bovine granulocytes during proliferation and maturation in the bone marrow. Therefore, functions and receptors playing a crucial role in udder defense and found to be deficient during periparturient *E. coli* mastitis were studied. The Fc-IgG₂ receptor and CD11b expression, the occurrence of phagocytosis, the respiratory burst activity, and the cellular myeloperoxidase activity were studied in three fractions of granulocytes with different maturation characteristics.

MATERIALS AND METHODS

Bone Marrow Samples

Bone marrow samples were collected by sternal aspiration in adult cattle at the slaughterhouse of Ghent University, as described previously (Van Merris et al., 2001b). Briefly, samples were taken within 15 min after killing. Before cleavage of the carcass, approximately 2 ml marrow was aspirated from the 3rd or 4th sternbrae. The first drop of each aspirate was used for a control smear. Bone marrow samples were transferred into sterile tubes containing 5 ml Iscove’s Modified Dulbecco’s Medium (Gibco BRL, Life Technologies Inc.), supplemented with 10% fetal bovine serum (FBS; Gibco BRL) and 100 U/ml lithium heparin (Van Merris et al., 2001a).

Density Gradient Separation

Bone marrow cells were fractionated into different stages of maturation using a three step Percoll gradient originally described by Cowland and Borregaard (1999) for the human, and adapted for the bovine by Van Merris et al. (2001a). Briefly, the marrow suspension was layered on top of a discontinuous three-layer Percoll gradient with increasing densities, 1.030, 1.060, and 1.080 g/ml respectively. Following centrifugation, three cellular bands were selectively retrieved: fractions I, II, and III. To characterize each fraction, cell

suspensions were submitted to a forward scatter/side scatter (FS/SS) dotplot analysis in the flow cytometer. Smears were analyzed visually under the light microscope. The fractions corresponded to three main maturation stages: early immature (fraction I), late immature (fraction II), and mature (fraction III). In each fraction, the viability of the cells was determined through the propidium-iodide (PI; Sigma Chemical Co, MO) exclusion test (Hoeben et al., 1997).

Fc-IgG₂ Receptor Expression

Fc-IgG₂ receptor expression was assessed by flow cytometry through direct labeling with fluorescein isothiocyanate (FITC) conjugated sheep anti-bovine IgG2 antibody (Serotec, Oxford, UK), as described by Worku et al. (1994). Each maturation fraction containing 1.10^6 cells in 100 μ l staining buffer [PBS supplemented with 1% FBS and 0.09% (w/v) sodium azide] was incubated with a saturating amount of the antibody for 30 min at 4°C in the dark. Cells were washed twice with 300 μ l staining buffer, pelleted by centrifugation ($200 \times g$, 10 min, 4°C) and resuspended in 400 μ l PBS. Subsequently, the fluorescence was measured by flow cytometry.

Fluorescence microscopy was also executed on the same samples for visualization of the Fc-IgG₂ receptor expression.

CD11b Expression

CD11b expression was assessed by flow cytometry through an indirect labeling technique. Each maturation fraction was adjusted to 1.10^6 cells in 100 μ l staining buffer. Cells were incubated with a saturating amount of mouse anti-bovine CD11b (Probio Ltd., Kent, England) for 30 min at room temperature. Cells were washed twice with 300 μ l staining buffer and pelleted by centrifugation ($200 \times g$, 10 min, 4°C). Pellets were incubated in the dark with a F(ab')₂ goat anti-mouse IgG conjugated to FITC (Sigma) for 30 min at 4°C. Cells were washed twice with staining buffer, and resuspended in 400 μ l PBS. This staining technique was previously used on circulating bovine PMN and resulted in the labeling of 95 to 99% of the cells (Dosogne et al., 1997).

For the determination of the total CD11b content, the primary bovine antibody was added both before and after the fixation and permeabilisation procedure (Lundahl et al., 1991), carried out with a Cytofix/Cytoperm fixation kit (PharMingen, San Diego). In this way, both cytoplasmic membrane expression and intracellular CD11b content were measured.

Fluorescence microscopy was also executed on the same samples for visualization of the CD11b expression.

Phagocytosis of *Escherichia coli*

To test phagocytic capacity, the method described by Hoeben et al. (1997) was used. In this method, phagocytosis is tested without discrimination between attachment and ingestion of *E. coli* bacteria. Following isolation, cell suspensions were analyzed by flow cytometry FS/SS dotplot to assess the percentage of granulocytic cells within each fraction (Van Merris et al., 2001a). The three fractions were adjusted to 1.10^6 granulocytic cells in 100 μ l HBSS (Gibco BRL). In chronological order, PI-labeled *E. coli* bacteria were added to the samples at a ratio of 1:25 and incubated in a shaking bath at 37°C with 5% autologous plasma. Phagocytosis was stopped after 20 min by addition of 0.1 mM N-ethylmaleimide (Sigma Chemical Co) in the same chronological order. Samples were further diluted with 400 μ l PBS, stored for a few minutes on ice and immediately analyzed by flow cytometry. Using this method, both non-immunological (i.e. mediated by complement, hydrophobic properties, or lectins) and immunological (i.e. antibody-dependent) phagocytosis was detected.

Fluorescence microscopy was also executed on the same samples to visualize the attachment and engulfment of *E. coli* bacteria.

Respiratory Burst Activity

The respiratory burst activity was measured as described by Smits et al. (1997), using dihydrorhodamine as substrate. Following isolation, cell suspensions were analyzed by flow cytometry in a FS/SS dotplot to assess the percentage of granulocytic cells within each fraction (Van Merris et al., 2001a). Subsequently, the three fractions were adjusted to 1.10^6 granulocytic cells in 100 μ l HBSS and incubated with 5% autologous plasma and 10 μ M dihydrorhodamine 123 (Molecular Probes, Leiden, The Netherlands) in a shaking bath at 37°C for 5 min. Samples were further diluted with 400 μ l PBS and the spontaneous reactive oxygen species (ROS) production was measured by flow cytometry. The stimulated respiratory burst activity was determined by adding 10 μ l 0.1 μ M phorbol 12-myristate 13-acetate (PMA; Sigma Chemicals Co) during 15 min, following the first incubation.

Cellular Myeloperoxidase Activity

Myeloperoxidase (MPO; EC 1.11.1.7) positive granules were microscopically analyzed in each maturation

fraction by staining the slides with benzidine dihydrochloride (Sigma Chemical Co) (Metcalf et al., 1986).

Flow Cytometric Analysis

Fluorescence was measured using a FACScan flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, California) equipped with a 488 nm argon-ion laser. Percent fluorescence (**F**) and mean fluorescence intensity (**MFI**) of selectively gated granulocytic cells in each maturation fraction (Van Merris et al., 2001a) were used to quantify the receptor expression and the functional activity of bone marrow cells. Percent fluorescence is defined as the percentage of granulocytic cells with a fluorescence higher than autofluorescence, attributed to respectively receptor expression (Fc-IgG₂ receptor, and CD11b), engulfment of bacteria (phagocytosis) or ROS production (respiratory burst). MFI was used as an index for the average number of receptors expressed, bacteria ingested or ROS produced per cell. For CD11b expression, the MFI was corrected by subtraction of background fluorescence obtained using an isotype-matched antibody control.

Differential Counting and Microscopic Analysis

The distribution of granulocytic precursors in the three maturation fractions was assessed by differential counting of 100 cells in each fraction. Morphologic identification of bovine bone marrow cells was carried out according to Wilde (1964). Cell suspensions were adjusted to a concentration of $1 \times 10^5 \text{ ml}^{-1}$ in PBS with 10% FBS. For light microscopy, 200 μl aliquots were centrifuged onto FBS-coated slides in a cytospin (Shandon, Runcorn, UK). The slides were air dried at room temperature, fixed in methanol and stained with Hemacolor (Merck Diagnostica, Darmstadt, Germany). Fluorescence microscopy was used as a qualitative control for all properties measured with flow cytometry. Cell suspensions were fixed in 1% paraformaldehyde, incubated in the dark with a nuclear dye (DAPI; Molecular Probes) for 20 min and washed twice with PBS prior to cytocentrifugation.

Statistical Analysis

The analyses were computed using statistical software (Statistix 4.1 NH Analytical Software). The normality of the data was tested using the Wilk-Shapiro/rankit plot. The significance of differences in receptor expression or functional activity of the bone marrow cells in the three maturation stages (fraction I, II, III) was determined by a LSD (t) pairwise comparisons of means. Data were expressed as means \pm SEM.

RESULTS

Myeloid Isolation and Viability

Density gradient centrifugation of bovine bone marrow suspension revealed three enriched fractions: early immature (fraction I), late immature (fraction II), and mature granulocytic cells (fraction III). Fraction I contained mainly myeloblasts and promyelocytes, fraction II consisted of myelocytes and metamyelocytes, and fraction III contained mainly band and segmented cells (Figure 2). Although fractions were not entirely pure, they proved to be useful to study the development of some PMN functions. Table 1 shows the differential count in the three maturation fractions. The viability of cells was found to be $86.4 \pm 2.28\%$ in fraction I, $95.3 \pm 1.33\%$ in fraction II and $98.1 \pm 0.78\%$ in fraction III ($n = 39$).

Fc-IgG₂ Receptor Expression

Fc-IgG₂ receptor was first detected in myeloblasts and promyelocytes ($22.2 \pm 2.58\%$ F in fraction I). The percent fluorescence increased strongly upon maturation, with $49.0 \pm 4.06\%$ fluorescence in the late immature fraction (fraction II, $P < 0.001$). Fc-IgG₂ receptor reached a maximal expression at the band and segmented stages ($73.3 \pm 4.24\%$ F in fraction III, $P < 0.001$). Moreover, MFI increased significantly ($P < 0.001$) with each subsequent maturation stage.

Results were obtained on eight cows and are summarized in Table 2.

CD11b Expression

CD11b was expressed on the cytoplasmic membrane of the granulocytic cells at all maturation stages (Figure 3). This complement receptor was first detected on promyelocytes ($3.71 \pm 0.49\%$ F in fraction I). A small increase was observed in the percent CD11b positive cells until the metamyelocytic stage ($13.4 \pm 2.58\%$ F in fraction II, $P < 0.001$). The number of cells expressing CD11b was found to be highest in the mature cells ($76.6 \pm 3.07\%$ F in fraction III). Band and segmented cells showed a six-fold increase in percent fluorescence in comparison with the late immature cells ($P < 0.001$). In contrast, a similar MFI value was observed for the three maturation fractions.

Following permeabilisation, the percent fluorescence in the early immature cells (fraction I) did not differ from the cytoplasmic membrane expression. However, an increase of 80% fluorescence was observed in the late immature fraction ($69.6 \pm 5.41\%$ F in fraction II, $P < 0.001$). Also in the mature fraction, more cells stained positively upon permeabilisation (increase of 12%, $P <$

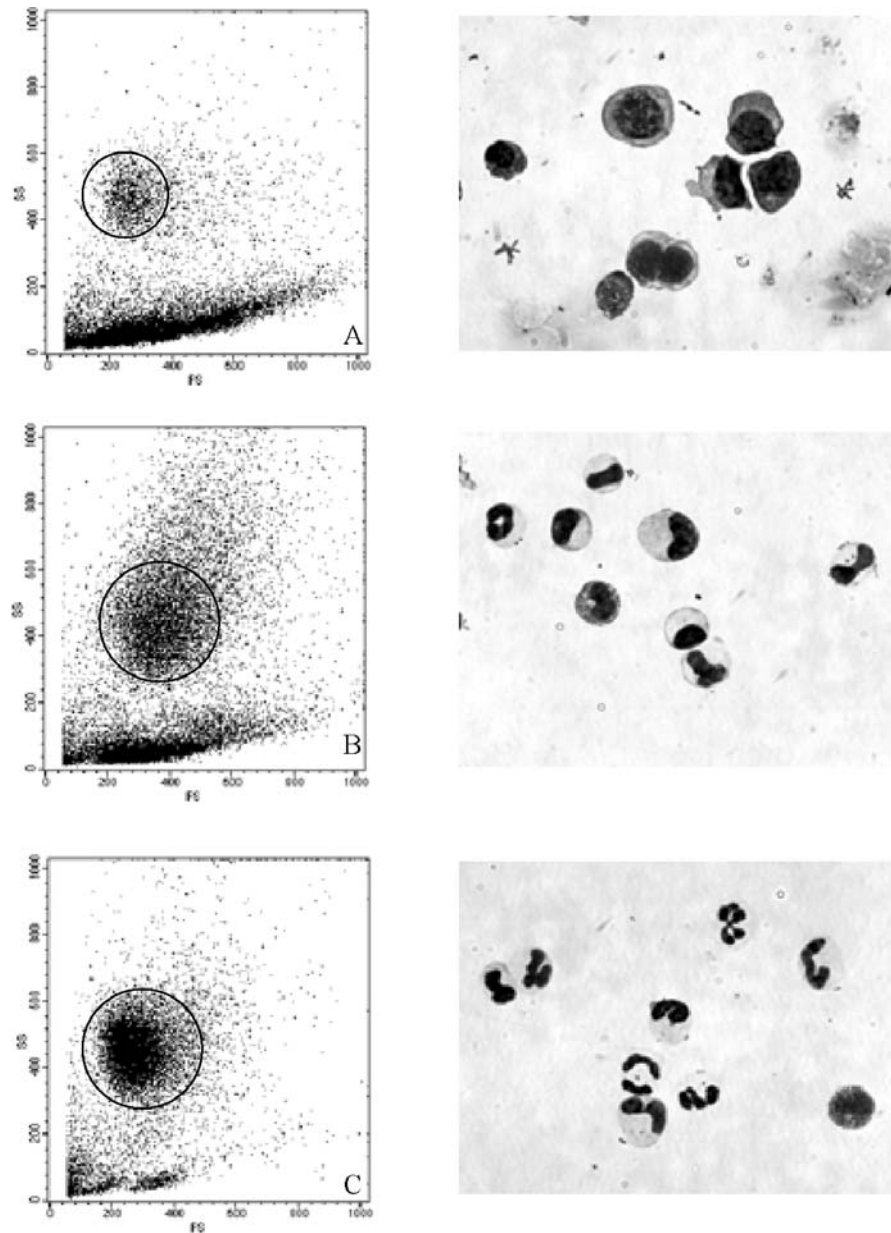


Figure 2. FS/SS dotplots and smears of the corresponding gated cells, obtained by cell sorting, in fraction I (A), fraction II (B), and fraction III (C). Figure was published previously by Van Merris et al. (2001a).

Table 1. Distribution of granulocytic precursors in three maturation stages (fractions I, II, III). Numbers refer to the mean percentage (\pm SEM) of the different maturation stages in each fraction, based on a differential count of 100 precursor cells ($n = 39$).

Fraction	Myeloid maturation stage		
	Myeloblasts, promyelocytes	Myelocytes, metamyelocytes	Band cells, segmented cells
I	84.0 \pm 1.87	9.51 \pm 2.02	6.62 \pm 1.31
II	5.10 \pm 1.35	88.1 \pm 2.12	6.95 \pm 1.36
III	1.0 \pm 0.0	4.77 \pm 1.35	95.2 \pm 1.34

Table 2. Percent Fc-IgG₂ positive cells (% F) and their mean expression (MFI) in fractions I, II, and III. Data are expressed as means \pm SEM (n = 8).

Fraction	%F	MFI
I	22.2 \pm 2.58 ^a	22.3 \pm 3.36 ^b
II	49.0 \pm 4.06 ^a	44.6 \pm 2.00 ^b
III	73.3 \pm 4.24 ^a	103 \pm 9.49 ^b

^{a,b}*P* < 0.001.

0.001). MFI in fractions I, II and III increased with 60% (*P* < 0.001) compared to the value prior to permeabilization.

All results were obtained on nine cows and are summarized in Table 3 and Figure 3.

Phagocytosis of *Escherichia coli*

Early immature cells did not attach nor ingest *E. coli*. The small percent fluorescence (1.37 \pm 0.28% F in fraction I) was due to the presence of more mature cells, as demonstrated with the fluorescence microscope. Some late immature cells showed a minimal phagocytic ability (3.90 \pm 0.43% F in fraction II, *P* < 0.01). Band and segmented cells were the most active phagocytes showing substantial phagocytic capacity. Nevertheless, only 46.2 \pm 3.65% of the mature population was phagocytizing in vitro (*P* < 0.001).

Results were obtained on 11 cows and are summarized in Table 4.

Respiratory Burst and Cellular Myeloperoxidase

Early immature cells did not show respiratory burst activity. The small percent fluorescence (2.35 \pm 0.39% F in fraction I) was attributed to more mature cells contaminating this fraction. Within the late immature fraction (fraction II) 9.64 \pm 1.38% of the cells produced ROS (*P* < 0.001). In the mature fraction a significantly

higher percent fluorescence could be detected (36.6 \pm 2.69% F in fraction III, *P* < 0.001). The MFI was not significantly different between fractions I, II and III.

Following PMA stimulation, no differences in percent fluorescence could be detected in early and late immature fractions compared to the basal ROS production. However, the MFI increased three-fold in the late immature fraction following stimulation (fraction II, *P* < 0.001). On average 57.1 \pm 2.70% fluorescence was detected in band and segmented cells (fraction III). The MFI showed a ten-fold increase in comparison to the spontaneous burst levels (*P* < 0.001).

All results were obtained on 11 cows and are summarized in Table 5 and Figure 5.

Myeloperoxidase positive granules were observed as discrete blue granules in the cytoplasm of granulocytic cells at all maturation stages, starting with the promyelocyte.

DISCUSSION

The neutrophils of the bovine play a key role in the defense against udder infection, and impaired functional response has been proposed to contribute to the pathogenesis of clinical mastitis (Burvenich et al., 1994). The importance of cellular maturity in the defense against intramammary infections is supported by the correlation between the severity of mastitis and the total number of circulating neutrophils immediately before infection (Heyneman et al., 1990; Kremer et al., 1993; Vandeputte-Van Messom et al., 1993; Dosogne et al., 1997). Cows with a low number of circulating neutrophils become more severely diseased during subsequently induced *E. coli* mastitis.

The knowledge about the acquisition of functional properties during bovine granulopoiesis is based on studies in human bone marrow (Glasser and Fiederlein, 1987). There is only little information about the functional differentiation of bovine cells. A more in depth

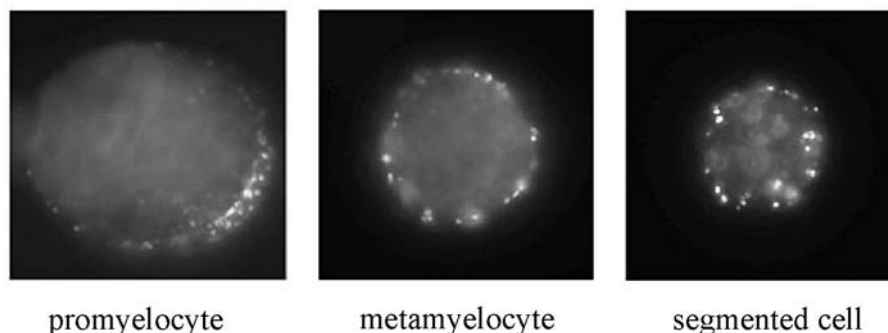


Figure 3. CD11b expression through fluorescence microscopy (original magnification at 630 \times). Fluorescence is detected in the cytoplasmic membrane of bovine bone marrow cells of all maturation stages.

Table 3. Percent CD11b positive cells (% F) and their mean expression (MFI) on the cytoplasmic membrane, respectively the total content in fractions I, II, and III. Data are expressed as mean \pm SEM (n = 9).

Fraction	Cytoplasmic membrane expression		Total content	
	%F	MFI	%F	MFI
I	3.71 \pm 0.49 ^a	63.0 \pm 4.41 ^{ns}	2.80 \pm 0.46 ^d	150 \pm 7.98 ^e
II	13.4 \pm 2.58 ^{a,b}	61.7 \pm 5.89 ^{ns}	69.6 \pm 5.41 ^{b,d}	155 \pm 6.67 ^e
III	76.6 \pm 3.07 ^{a,c}	58.2 \pm 1.31 ^{ns}	85.2 \pm 2.59 ^{c,d}	143 \pm 5.55 ^e

^{a,b,c,d} $P < 0.001$.^e $P < 0.05$.^{ns}Nonsignificant.

investigation of bovine bone marrow cells is therefore essential for the determination of the immune status in the bovine, because species variation in neutrophil function exists (Styrt, 1989).

The inflammatory reaction is initiated by extravasation of PMN (diapedesis). This is followed by a directed movement towards the site of infection (chemotaxis), where phagocytosis and killing of the pathogen will take place. Extravasation of activated neutrophils occurs after adhesion of the cells to the endothelial surface and is mediated by specific membrane adhesion molecules, e.g. the β_2 -integrin CD11/CD18. The entire process of phagocytosis entails recognition and adhesion, followed by internalization of the pathogen that will be degraded through oxygen-dependent and -independent mechanisms. In order for phagocytosis to proceed, neutrophils must recognize the bacteria. Immunological recognition is mainly accomplished by IgG₂ antibodies which recognize the bacteria through F(ab)-regions and bind to the neutrophils via Fc-receptors on their cytoplasmic membrane. Activation of complement also promotes phagocytosis. Therefore, CD11b functions as a

receptor for complement factor C3bi. Once complement and immunoglobulins bind to receptors on the neutrophil surface, the cell becomes activated and generates ROS. The respiratory burst system is very effective for eliminating Gram-negative bacteria such as *E. coli* (Burvenich et al., 1994). Moreira da Silva et al. (1998) observed a minimal respiratory burst of neutrophils three days after calving. This time period coincides with the maximum number of immature neutrophils found in circulation. The question arises whether the fall in the oxidative burst is the direct consequence of the appearance of immature cells in circulation.

Because neutrophils mainly constitute the first line defense in the udder, receptors and functions that play a role in the inflammatory reaction were studied during granulopoiesis: Fc-IgG₂ receptor and CD11b expression, complement mediated phagocytosis of *E. coli*, the respiratory burst activity, and the cellular myeloperoxidase activity.

In the present study the Fc-IgG₂ receptor was detected in bovine bone marrow as early as the myeloblasts and promyelocytes. The percent Fc-IgG₂ positive

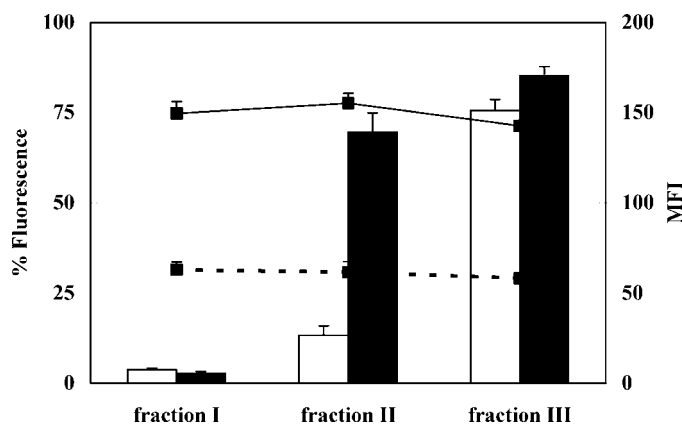
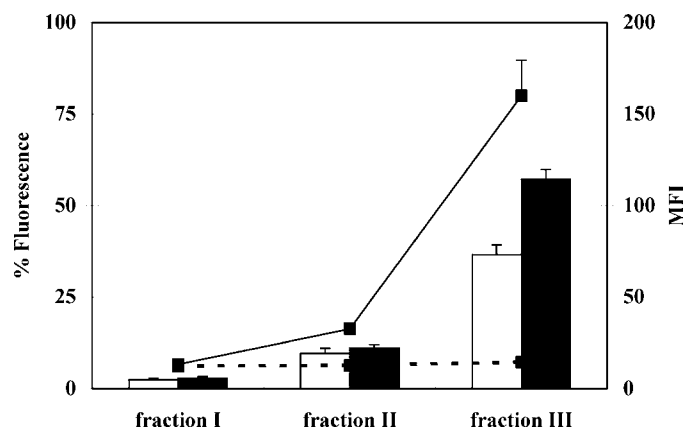
**Figure 4.** CD11b expression on bovine bone marrow cells: cytoplasmic membrane expression (\square : % F, and ---: MFI) and total CD11b content (\blacksquare : % F, and —: MFI) in fractions I, II, and III. Data are expressed as means and error bars represent SEM (n = 9).**Figure 5.** Respiratory burst activity in bovine bone marrow cells: spontaneous (\square : % F, and ---: MFI) and PMA-induced respiratory burst activity (\blacksquare : % F, and —: MFI) in fractions I, II, and III. Data are expressed as means \pm SEM (n = 11).

Table 4. Percent phagocytic cells (% F) and their mean activity (MFI) in fractions I, II, and III. Data are expressed as mean \pm SEM (n = 11).

Fraction	%F	MFI
I	1.37 \pm 0.28 ^{a,b}	20.3 \pm 2.47 ^d
II	3.90 \pm 0.43 ^{a,c}	24.6 \pm 2.12 ^d
III	46.2 \pm 3.65 ^{b,c}	40.4 \pm 1.91 ^d

^aP < 0.01.^{b,c,d}P < 0.001.

cells increased with the stage of maturation. The MFI was highest in the mature fraction, the immature cells having fewer binding sites for IgG₂. No comparative data for the bovine species are described in literature. Only one paper describes that the Fc-IgG₂ receptor was present on bovine neonatal blood PMN and that its expression was reduced in comparison to circulating PMN isolated from adult cattle (Zwahlen et al., 1992). On human granulocytic precursors, Fc receptors were also found to be expressed early in differentiation, more specifically at the promyelocytic stage (Glasser and Fiederlein, 1987).

We demonstrated that CD11b surface antigen was expressed in all maturation stages of bovine bone marrow cells. The CD11b expression on the cytoplasmic membrane increased significantly as precursor cells underwent granulocytic differentiation. However, in a comparable study on the characterization of bovine progenitors, CD11b was detected only on maturing cells, mostly myelocytes, metamyelocytes and segmented granulocytes (Fritsch et al., 1991). We propose that this discrepancy in results could be accounted for either by the difference in isolation procedure and/or the monoclonal antibody used. In humans, CD11b has been shown to be present in increasing amounts on cell surface starting from the myelocytic stage (Arnaout, 1990). Although Rosmarin et al. (1989) observed CD11b expression at low levels already in myeloblasts.

Additionally, we determined the total CD11b content in bovine bone marrow cells. In humans as well as in bovine, circulating PMN can increase their cytoplasmic membrane expression of CD11b upon stimulation

through mobilization of preformed CD11b, stored in specific granules. A significant intracellular CD11b pool was detected in bovine myelocytes, metamyelocytes, band and segmented cells.

The present results confirm that immature bovine cells do not exhibit a phagocytic ability. Progenitor cells started to attach and ingest *E. coli* bacteria at the myelocytic stage; only in band and segmented cells was detected a significant phagocytic capacity. Moreover, only 50% of these apparently mature granulocytes exhibited phagocytosis. This remarkable observation indicates that cellular characteristics required for optimal phagocytosis are acquired in conjunction with their release from the bone marrow (Altman and Stossel, 1974). Although CD11b is already expressed early in maturation, its presence does not guarantee complement mediated phagocytosis by immature bovine bone marrow cells. According to Lichtman and Weed (1972), immature cells may be less capable of forming pseudopods following phagocytic activation. Our findings are in general agreement with the observations of Silva et al. (1989), who studied immune phagocytosis in bone marrow cells of cows. However, Guidry and Paape (1976), inducing a left-shift in bovine following intramammary *E. coli* endotoxin infusion, observed that the immature peripheral granulocytes are equally phagocytic as their mature counterparts. Decreased phagocytosis as compared to circulating PMN has also been demonstrated in human immature as well as morphologically mature bone marrow cells (Altman and Stossel, 1974; Glasser and Fiederlein, 1987).

Assessment of the respiratory burst activity demonstrated that immature cells isolated from bovine bone marrow were not capable to produce ROS, neither spontaneously, nor after stimulation. Following PMA-stimulation, myelocytes and metamyelocytes showed a significant respiratory burst activity, whereas they did not exhibit any spontaneous ROS production. The band and segmented cells on the contrary had a spontaneous respiratory burst activity and PMA induced a significant increase in ROS production.

Two enzymes play a keyrole in the ROS production: myeloperoxidase and the membrane-bound NADPH-

Table 5. Percent cells showing respiratory burst activity (% F) and their mean activity (MFI), with and without PMA stimulation in fractions I, II, and III. Data are expressed as mean \pm SEM (n = 11).

Fraction	Spontaneous respiratory burst		PMA-induced respiratory burst	
	%F	MFI	%F	MFI
I	2.35 \pm 0.39 ^a	12.3 \pm 1.92 ^{ns}	2.77 \pm 0.47 ^c	13.3 \pm 1.71 ^f
II	9.64 \pm 1.38 ^a	13.6 \pm 2.02 ^{ns,d}	11.1 \pm 0.96 ^c	32.7 \pm 2.21 ^{d,f}
III	36.6 \pm 2.69 ^{a,b}	14.4 \pm 2.14 ^{ns,e}	57.1 \pm 2.70 ^{b,c}	160 \pm 19.5 ^{e,f}

^{a,b,c,d,e,f}P < 0.001.^{ns}Nonsignificant.

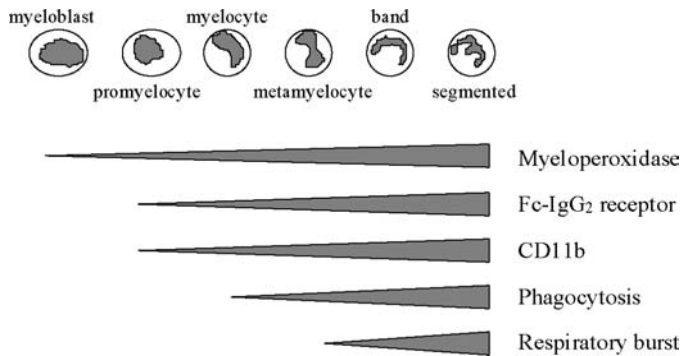


Figure 6. Proposed stepwise acquisition of various functional properties in bovine bone marrow cells during granulopoiesis (adapted from Glasser and Fiederlein, 1987).

oxidase. Myeloperoxidase is found exclusively in azurophilic granules and is an abundant early myeloid protein (Rosmarin, 1989). Also in the present study on bovine bone marrow cells, MPO positive granules were found at all maturation stages, starting with the promyelocyte. Following our results, we suggest that only from the metamyelocytic stage, bovine granulocytic cells dispose of a functionally oxidative burst. Comparative data for the bovine species are scarce. In a study conducted by Silva et al. (1989), the nitroblue tetrazolium reduction, an alternative parameter for ROS production, was only observed in segmented granulocytes from the bovine bone marrow. Also in human bone marrow, only band and segmented cells showed a substantial oxygen-dependent microbial killing using nitroblue tetrazolium reduction (Glasser and Fiederlein, 1987). The minor differences in the data could be attributed to the different techniques used for assessing the ROS production.

CONCLUSION

In conclusion, the maturation sequence of the functional properties acquired during bovine granulopoiesis was studied. According to our results, the following sequence of events throughout the maturation of bovine bone marrow cells is proposed: myeloperoxidase activity → Fc-IgG2 receptor expression → CD11b expression → complement mediated phagocytosis → respiratory burst activity (Figure 6). Compared to the functional differentiation of human neutrophils, no major differences are noticed, with exception of phagocytosis. In human bone marrow, even a rare blast cell had the capacity to phagocytize (Glasser and Fiederlein, 1987), whereas bovine precursor cells started to be phagocytic only at the myelocytic stage.

A more detailed analysis of function and receptor expression is necessary to relate and quantify cell type

and functional maturity. However the present study has shown that a simple Percoll fractionation is sufficient to demonstrate the functional maturation sequence of bovine progenitor cells. Our findings support the hypothesis that the release of immature cells from the bone marrow into circulation, after intramammary infection, may contribute to the decrease of a number of important PMN functions. Moreover, a suppression of the granulopoiesis at the level of the bone marrow during the periparturient period was hypothesized by Burvenich et al. (1994). Because on one hand, the number of circulating neutrophils largely depends on the proliferative activity of the bone marrow, and on the other hand the number of circulating neutrophils and their maturity after parturition is of major importance for the defense against mastitis (Moreira da Silva et al., 1998), further investigations are strongly suggested.

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